Improved Methods for the Synthesis of Nucleic Acids

The present invention relates to improved methods for the synthesis of nucleic acids, comprising the incubation of a polymerase, a nucleic acid that can serve as a template for the polymerase, NTPs and Mn^{2+} under conditions enabling the synthesis of a nucleic acid strand wherein these conditions are characterised by comprising a molar ratio of Mn^{2+}/NTP of not more than 0.7.

The invention relates in particular to methods for the preparation of RNA, wherein DNA is used as a template and wherein at least a 1000-fold amplification rate is achieved. Further, the present invention relates to kits which comprise the components required for performing the methods according to the invention.

In vitro amplification of nucleic acids is necessary to perform a number of methods of molecular biology, for example cloning, sequence analysis, in vitro expression etc. Accordingly, methods were developed which permit in vitro synthesis of nucleic acids. In general, these procedures can be distinguished on the basis of the reaction product, which is either DNA or RNA.

In vitro transcription is a method to synthesise RNA, usually utilising double-stranded DNA as a template. In this method, isolated components of the cellular transcription machinery (RNA polymerase and NTPs) are used for an enzymatic reaction, performed in a reaction tube. It is assumed, that a complex of Mg^{2+} and NTP is the substrate for the synthesis reaction.

Therefore, Mg^{2+} is an essential component of the reaction and it is usually added in excess in relation to the NTP concentrations (Milligan and Uhlenbeck, Methods in Enzymology, Vol. 180 (1989), 51-62; and Wyatt et al., Biotechniques, Vol. 11 (1991),764-769).

To optimise the amplification rate of the in vitro transcription, e.g. US 5,256,555 suggests the use of a total nucleotide concentration of more than 16 mM. At the same time, Mg^{2+} that is essential for the reaction, should be used in a concentration that is not higher than 10% above the total concentration of all nucleotides. Furthermore, inorganic pyrophosphatase should be present in the reaction.

The best known method for DNA synthesis is the polymerase chain reaction (PCR), developed by Kary Mullis in the mid-eighties (see Saiki et al., Science, Vol. 230 (1985), 1350-1354; and EP 201 184).

During the PCR reaction, single stranded primers (oligonucleotides with a chain-length of usually 12 to 24 nucleotides) anneal to a complementary, single stranded DNA sequence. The primers are elongated by a DNA polymerase and the deoxyribonucleoside triphosphates (dNTPs, i.e. dATP, dCTP, dGTP, dTTP), to obtain a double stranded DNA. The double stranded DNA is separated by heating into single strands. The temperature is reduced sufficiently to permit a new step of primer annealing. Again, primers are elongated by DNA polymerase to obtain a second DNA stand.

Repetition of the steps described above enables the exponential amplification of the DNA template strands. This is achieved by adjusting the reaction conditions such that almost each reaction cycle results in the conversion of each DNA single strand to a double strand, subsequently separated again in two single strands which are used again as template for further strands.

If a reverse transcription reaction is performed prior to this method, wherein an mRNA is converted to a single stranded DNA (the so-called cDNA) by means of an RNA-dependent DNA polymerase, then the PCR reaction can directly be applied for amplification of nucleic acids starting from an RNA sequence (see EP 201 184).

In addition to the mentioned basic reaction schemes, a multitude of alternatives have been developed, that can be distinguished based on the starting materials (RNA, DNA, single or double stranded) and the reaction product (amplification of specific RNA or DNA sequences from one sample, or the amplification of all sequences).

Both, the patents DE 101 43 106.6 and DE 102 24 200.3 describe methods for the amplification of ribonucleic acids, that comprise a combination of individual steps of the PCR reaction and a transcription reaction.

Despite the above advances, there is still a need for further improvement of methods for the synthesis of nucleic acids, in particular for methods that permit a high synthesis rate and synthesis yield in combination with a low consumption of chemicals.

This problem has now been solved by providing a method for the synthesis of nucleic acids, comprising the incubation of a polymerase, a nucleic acid that can serve as a template for the polymerase, NTPs and Mn^{2+} under conditions that permit the synthesis of a nucleic acid strand, **characterised in that** the conditions comprise a molar ratio of Mn^{2+}/NTP of not more than 0.7.

Surprisingly, the method of the present invention was able to show that already the choice of the above mentioned molar ratio of Mn^{2+}/NTPs can result in a significantly increased synthesis rate of the polymerase. At the same time, the method according to the invention is more cost-efficient, because lower NTP concentrations are used. Therefore, a high amplification rate can be achieved by simple and cost-efficient means.

In the context of the present invention, the term "molar ratio of Mn^{2+}/NTP" is used to provide a numerical value for the quotient of the molar concentration of Mn^{2+} in relation to the total molar concentration of all NTPs.

In an especially preferred embodiment of the method according to the invention, the synthesis conditions are chosen that the a molar ratio of Mn^{2+}/NTP is between 0.2 and 0.6, preferably between 0.3 and 0.5.

Using the molar ratios according to the invention may result in a total NTP concentration between 4 mM and 24 mM; the use

of 4 different NTPs results in concentrations between 1 mM and 6 mM for each NTP.

This provides a preferable Mn^{2+} concentration range between 0.8 mM (molar ratio of 0.2 and total NTP concentration of 4 mM) and 14.4 mM (molar ratio of 0.6 and total NTP concentration of 24 mM).

Any polymerase can be used in the method according to the invention. However, the use of an RNA polymerase is preferred for all embodiments of the invention, and in particular a DNA dependant RNA polymerase that needs a DNA template having a promoter for the synthesis of RNA. For example a T7 RNA polymerase, a T3 RNA polymerase or an SP6 RNA polymerase may be used.

The RNA polymerase can either be an RNA-dependent or a DNA-dependent polymerase. Most naturally occurring DNA-dependent RNA polymerases can also use RNA as a template, if present in a suitable structure (see Konarska, M.M. and Sharp, P:A:, Cell Vol. 57 (1989),423-431; and Konarska, M.M and Sharp, P.A. Cell Vol. 63 (1990), 609-618).

The polymerase and the template nucleic acid have to be compatible. For example, the template nucleic acids used by an RNA polymerase have to comprise certain sequences or structures that are recognised by the RNA polymerase, and that permit initiation of the synthesis. Preferably, DNA is used as a template for the RNA polymerase. The corresponding DNA may contain a promoter region, that is recognised by the RNA polymerase and that is used for the start of the synthesis. Alter-

natively, the DNA may contain other structures, which are recognised by the RNA polymerase and allow initiation of the synthesis. Such alternative structures are described in Krupp (Nucleic Acid Res. Vol. 17 (1989), 3023-3036) and in Kuhn et al. (Nature Vol.344 (1990), 559-562).

Because the method according to the present invention results in a very high amplification rate, the template nucleic acid can be used in very low concentration. For example, the template can be DNA or mRNA and it is used in an amount of at least 0.1 picogram, or 0.2 attomol, respectively. In a reaction volume of 20 µl this results in a minimum concentration of 10 femtomolar.

The reaction mix has to contain NTPs. RNA polymerase is usually used in combination with ATP, UTP, CTP, and GTP. In the conventional transcription reaction according to the state of the art, all mentioned NTPs are used in a reaction. However, it can be desired or useful, to use only one or a few of the NTPs.

Alternatively, or in addition to the NTPs, dNTPs can be used in the method according to the invention combined with the use of an RNA or a DNA polymerase. This way of proceeding has the special advantage that the transcript has complete or partial DNA characteristics, i.e. it becomes nuclease resistant and serves as a better template for the RNA polymerase. Commonly used dNTPs are dATP, dTTP, dCTP and /or dGTP.

All or some of the NTPs and/or of the dNTPs can be used as modified compounds or derivatives. According to the state of

the art, commonly used derivatives include coupling to biotin or to a fluorescence label, e.g. for simplifying the detection of the synthesis products.

The reaction time and further reaction conditions (temperature, pH and so forth) are easily chosen by one skilled in the art, and they depend on the polymerase used and the expected amplification rate. The incubation time, for example, can range between 1h and 24h; however, preferably between 4h and 16h. If the T7 RNA polymerase is used, an incubation temperature between 30°C and 45°C is the obvious choice.

The method according to the invention surprisingly provides an improved amplification rate. In the context of the present invention, the term "amplification rate" means the ratio of the amount of synthetically produced nucleic acids in relation to the amount of the input template. The method according to the invention permits an amplification rate of at least 1000, preferably at least 2000. Under optimal reaction conditions, even an amplification rate of 2500 was achieved.

The methods according to the invention can be used for a multitude of applications. For example, the improved methods for the production of nucleic acids can be used in the methods for amplification of ribonucleic acids as described in DE 101 43 106.6 and DE 102 24 200.3. The nucleic acids obtained according to the method of the invention can be coupled to a chip and used as probes. The methods can be used for the in vitro transcription, for the analysis of interactions with nucleic acid binding factors, as aptamers for specific binding of molecules, as ribozymes, etc.

Finally, the present invention relates to kits for the synthesis of nucleic acids, which comprise one or more containers, containing a polymerase, NTPs, dNTPs and/or their derivatives (for example NTPs or dNTPs that are biotinylated or coupled to a fluorescence label) and Mn^{2+}. Preferably, the polymerase is an RNA polymerase, preferably a DNA dependant RNA polymerase that needs a DNA template having a promoter to synthesize RNA. Especially preferred is the use of the T7 RNA polymerase, the T3 RNA polymerase, or the SP6 RNA polymerase.

Preferably, such kits further contain instructions for performing one of the methods according to the invention. Such instructions or manuals will describe in full detail the amounts of the different reaction components that have to be mixed to obtain optimal synthesis results.

Short description of the Figures

Fig.1 In vitro transcription using different concentrations of Mn^{2+} and Mg^{2+} and NTPs with a total concentration of 16 mM. Determination of the optimal Mn^{2+}/NTP ratio and comparison with Mg^{2+}.

Fig.2 Determination of the optimal NTP concentration, using various Mn^{2+}/NTP ratios.

Fig.3 Determination of the amplification rate.

Example 1

In this example, the transcription rates of the RNA polymerase were determined, dependent on various concentrations of Mn^{2+} as well as Mg^{2+}.

For this purpose, a 20µl reaction volume, the components 40 mM Tris-HCl (pH 8), 10 mM DTT, 2 mM spermidine, 0.01% Triton X-100, 10 ng of a nucleic acid template (plasmid pTRI-Xef), 10 U RNasin (RNase-inhibitor), 40 U T7 RNA polymerase, 4 mM NTPs (each; resulting in a total of 16 mM), and Mn^{2+} or Mg^{2+} in a concentration ranging from 4 mM to 10 mM were pipetted together and were incubated for 16h.

Aliquots of 5µl were withdrawn and separated by means of electrophoresis on a 1% agarose gel, and photographed after staining with ethidium bromide. The result is shown in **Fig. 1**. Dependant on the concentrations of Mn^{2+} and Mg^{2+}, respectively, the resulting ratios of Mn^{2+}/NTPs vary between 0.25 and 0.625.

Fig. 1 clearly shows for all reaction conditions, that the presence of Mn^{2+} resulted in a better amplification rate, as compared with Mg^{2+}.

Example 2

The aim of this example was to determine the optimal NTP concentration, dependent on the Mn^{2+}/NTP ratio.

For this purpose, a series of in vitro transcription reactions were performed, as outlined in example 1. The concentration of NTPs ranged from 2 mM to 10 mM for each NTP, and the $MnCl_2$ con-

centrations were between 2.4 mM and 24 mM, resulting in a Mn^{2+}/NTP ratio between 0.3 and 0.6.

The amount of transcript obtained (in ng) was determined by staining the gel with ethidium bromide and the use of an RNA dilution series as reference.

The result is summarised in **Fig. 2** and shows, that already at a concentration of 4 mM of each NTP (total concentration of NTPs was 16 mM) resulted in a maximal synthesis rate. The best results were obtained using a combination of 4 mM of each NTP and 6.4 mM $MnCl_2$ (corresponds to a ratio of 0.4)

Example 3

In this example, the amplification rate was determined in relation to the incubation time. For this purpose first of all, an in vitro transcription reaction as described in example 1 was set up, wherein 4.8 mM $MnCl_2$ and 4 mM NTP (total of 16 mM) were used (corresponds to a Mn^{2+}/NTP ratio of 0.3).

At the time points indicated in **Fig. 3**, aliquots of 5µl were withdrawn and analysed on a 1% native agarose gel. The results are shown in **Fig. 3** and they reveal, that an amplification factor of more than 1500 was obtained in all reaction conditions.